(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) Publication number: **0 075 353**
A1

(12) **EUROPEAN PATENT APPLICATION**

(21) Application number: 82201107.8

(22) Date of filing: 08.09.82

(51) Int Cl³: G 01 N 21/75, G 01 N 33/54

(30) Priority: 18.09.81 EP 81810385

(43) Date of publication of application: 30.03.83 Bulletin 83/13

(84) Designated Contracting States: AT BE CH DE FR GB IT LI LU NL SE

(71) Applicant: BATTELLE MEMORIAL INSTITUTE, 7 route de Drize, CH-1227 Carouge/Genève (CH)

(72) Inventor: Carter, Timothy, Flat No. 5 - Croft House Parsonage Street, Halstead CO92LB Essex (GB)
Inventor: Dähne, Claus, 103, Avenue du Bois de la Chapelle, CH-1213 Onex (CH)
Inventor: Place, John F., 42 Chemin de la Chevillarde, CH-1208 Geneva (CH)

(74) Representative: Doussa, Blanco et al, 7, route de Drize, CH-1227 Carouge/Genève (CH)

(54) Method for the determination of species in solution with an optical wave-guide.

(57) An analyte in solution is made to react with a specific reactant coated on the wave-guide thus modifying the optical properties thereof. The index of refraction of the wave-guide material is higher than that of the reaction medium which ensures that a light signal injected into said guide be carried by multiple total reflection, the distance of penetration of the evanescent wave associated with the totally reflected signal being of the same order of magnitude or greater than the thickness of the analyte-reactant product layer.

EP 0 075 353 A1

SRU 000882

0075353

METHOD FOR THE DETERMINATION OF SPECIES IN SOLUTION WITH AN OPTICAL WAVE-GUIDE

The present invention relates to the use of waveguide for the determination of the concentration of a species (or analyte) in solution in a liquid by measuring the rate (concentration dependent) of its combination with or dissociation from a specific reactant thereto, e.g. a conjugate moiety in a complexation reaction. More specifically, the invention concerns a method for determining an analyte in solution in which a layer of analyte-reactant product is formed at the surface of a lit waveguide carrying a totally and multiply reflected electromagnetic wave signal and changes the optical properties thereof so as to modify said signal, said modification being measured and used for said determination. Thus, the invention applies to a variety of chemical and biological systems and, particularly well, to the determination of bioactive molecules in low concentration by immunoassay type reactions, i.e. reactions based on the formation of complexes by the addition of antibody (AB) to antigen (AG) molecules or vice-versa.

Many methods already exist in the field for achieving the above mentioned determination based on the classical techniques of biochemistry. For instance, chemical reactions can be used to detect a given analyte in a number of different ways. Classical systems include titration or reaction with a specific reagent that gives a colored product or precipitate. The requirement for this detection system is that the reagent is in equivalence or in excess, so that the product can be measured by conventional photometry, turbidimetry, colorimetry, etc.. The measuring system is chosen according to the size of the signal to be measured. At very low analyte concentrations, detection becomes difficult and greater discrimination can be obtained, for example, by concentrating the reaction product locally e.g. by solvent extraction, centrifugation, etc.. which may become tedious and costly. However, the above disadvantage was strongly reduced when a practical system for the measurement of biochemical analytes in

extremely low concentrations was made available in 1960. This micro-analytical system (radioimmunoassay) took advantage of the characteristics of biological systems for molecular recognition (antigen-antibody reactions) and the extreme sensitivity of radioactive measurements (radioactive isotope labelling). An essential feature of this breakthrough was the concept of limited reagent assay with the tracer label used to measure the distribution of the analyte to be measured between the reagent-bound and the free moieties (see for example: Review Paper "The theoretical aspects of saturation analysis" R.P. EKINS in "In vitro procedures with radioisotopes in medicine", International Atomic Energy Agency, June 1970). Although immunoassays were first described as limited reagent assays, equally practical systems were later described for reagent excess methods (see MILES et al. Biochem. J. 108, 611 (1968).

In addition to volumetric and gravimetric analysis, the present methods thus involve highly sensitive methods such as colorimetry, spectroscopy and radioactive measurements. However, many of such techniques are now becoming obsolete as they are tedious, require a relatively large quantity of analyte to be accurate, are based on hard to prepare and difficult to store reagents or require expensive and cumbersome equipment and highly skilled operators. Thus, there is a trend now to develop more subtle methods, which require less reagents and which can be performed safely, quickly and accurately by moderately skilled personnel. Among such methods which have been disclosed lately, some involve the use of optical wave guides including the reactant. For analysis, the wave guide is contacted with the analyte in solution whereby a reaction with the reactant on the wave guide occurs with the consequence that the optical properties of the latter are modified. The measurement of such modification then provides the required data for the analyte determination. According to the teaching of some recent references (for instance, USP 4,050,895 (HARDY) and WO 81 100 912 (BUCKLES), the guides can consist (BUCKLES) of a porous light transmitting core impregnated with the reactant into which the analyte will diffuse during the reaction. Or, (BUCKLES or HARDY) the wave-guide can consist of a non porous light transmitting core (e.g. glass) coated with a porous or permeable sheath impregnated with the reactant and into which the analyte will dif-

fuse. Furthermore, in one specific case applied to immunoassay (HARDY, Example 3), a rod-shaped wave-guide is coated with an antibody layer bonded by diphenyldimethoxysilane and reacted with polystyrene latex spheres treated with an antigen. The antigen treated beads will then attach to the guide and modify the light signal output of the latter, which variation is used for the analytical determination.

The above techniques have merit but they do not well apply to some typical analyses involving reaction kinetics. Indeed, it is well known that rates may provide essential analytical data, particularly in the case of automated test systems and, since reactions occurring within permeable or porous bodies always involve a preliminary diffusion of the analyte into said body, and since diffusion processes are generally much slower than chemical reactions, the rate of the latter cannot be measured directly; in such a case, only equilibrium data can be obtained. Also in the known prior-art, embodiments are avoided involving the use of a transparent core coated with a reactant sheath with a refraction index smaller than that of the core for the reason that, admittedly, low sensitivity would be expected to result. Indeed in such case, most of the light signal injected at the input of the guide will travel within the core by a total reflection process and, in such case, as is commonly accepted, the interaction of that signal with the reaction products located in the sheath, i.e. outside the core should only be minor. Consequently, care was taken in the prior art that the refractive index of the sheath n_2 (where the reaction takes place) be always larger than that (n_1) of the core for allowing the light injected in the core at the input to be refracted into the sheath and, from that point on, to continue to travel in the sheath right to the output of the guide. Now, contrary to some of the prior disclosures, the output signal (the result of the light having been modified by passing through the products of reaction: reactant + analyte within the sheath) will not readily reenter the core and reach the back end thereof (this behavior results from elementary optical principles to be discussed later) and, for the measurements, the output light detector must be located in the very near vicinity of the testing probe (i.e. the back end of the sheath). Such arrangement is not always practical constructionwise, namely when the guide (plus sheath) is

dipped in a liquid for measuring an analyte in solution.

The present invention remedies these drawbacks as it involves no porous core or sheath and no diffusion through a matrix structure (sheath or core). Also, in the present invention, the light signal travelling inside the wave-guide by total reflection is neither transmitted by nor guided within the reactant analyte product; rather, only the evanescent wave component of the signal input (i.e. that part of the wave that extends into the region outside the core in the case of total reflection) is involved. Thus, since the range of action of the evanescent wave is only a fraction of wavelength (λ), the quantity of product needed (reactant + analyte) is extremely small and utmost sensitivity (with regard to the total amount of species to be analyzed) can be achieved.

Thus, one object of the invention is to provide a method for the fast and accurate determination, with an optical wave-guide, of the concentration of a chemical species or analyte in solution in a liquid. Another objection of the invention is to provide an analytical method for determining biological analytes with great specificity and sensitivity. Another object is to provide an analytical method which can be easily implemented by moderately skilled workers and which requires only a minor amount of analytical solution. Still another object of the invention is to provide versatile and automated measuring devices adapted for carrying out the above mentioned method.

These objects (and still further objects which will appear in the course of this description) are appropriately fulfilled by the present method which comprises using a wave-guide core, the index of refraction (n_1) of which is selected to be higher than that (n_2) of said analyte solution and to provide a ratio n_1/n_2 such that the depth of penetration in the solution of the electromagnetic field associated with said light signal travelling in the guide practically matches or exceeds the thickness of the said layer of analyte reactant product. Thus, for instance, the method involves contacting a section of a lit (non porous) wave-guide core coated with a thin film of a reactant specific to an analyte with a solution of said analyte thereby enabling said analyte to react with said reactant of the film and form a reactant-analyte product layer, observing the cor-

responding optical changes with time occurring to the light signal travelling through the core at the output of said core as the result of said product layer formation and comparing the rate data obtained with standard reference data obtained in a similar manner from calibrating samples of said analyte, the refractive index (n_1) of said core being greater than that (n_2) of said solution and the thickness of said film being only a fraction of the signal wavelength.

It will be useful at this stage to provide some general information on the transmission of light in a core by the so-called total reflection process. This can be better done with the help of part of the accompanying drawing in which:

Fig. 1 illustrates schematically the total reflection process of an incident beam N at the boundary between a dense (n_1) and a rare (n_2) medium at an angle θ larger than θ_c, the critical total reflection angle. In this figure, E_o is the initial magnitude of the electric field component of the light at zero depth in the rarer medium, Z is the depth of penetration axis and d_p is defined in the discussion below. R is the reflected beam.

Fig. 2 illustrates the fractional penetration depth of electromagnetic field in rarer bulk medium for total internal reflection versus angle of incidence for a number of interfaces. The penetration depth is infinitely large at the critical angle and is about one tenth the wavelength at grazing incidence for relatively high index media. $\lambda_1 = \lambda/n_1$ is the wavelength in the denser medium. (Taken from N.J. HARRICK, Internal Reflection Spectroscopy, Wiley 1967).

Fig. 3 illustrates the interaction of an evanescent wave and a layer of reaction product.

Physical insight into the interaction mechanisms at a reflecting surface can be obtained from a more fundamental approach with the aid of Maxwell's equations. In the present case, i.e. reflection in a dense medium at the boundary with a rare medium, the following question must be answered: what is the electromagnetic field distribution in the rarer medium beyond the reflecting interface for total internal reflection?

In this case, there exists a wave function in the rarer medium which propagates parallel to the interface. Its electric field amplitude falls off exponentially with distance from the surface (see

Fig. 1); therefore it is called an evanescent wave. In the ideal case and if the rarer medium has no absorptive property of its own at the wavelength considered, there is no net flow of energy into the non absorbing rarer medium, since the time average of the energy described by Poynting's vector (see, for instance M. BORN & E. WOLF, "Principles of Optics" Pergamon Press (1959)) is zero. Mathematically, the electric field can be described by the exponential function:

$$E = E_0 . e^{-\frac{Z}{dp}}$$

The depth of penetration d_p, defined as the distance required for the electric field amplitude to fall to e^{-1} of its value at the surface, is given by

$$d_p = \frac{\lambda_1}{2\pi (\sin^2\theta - n_{2-1}^2)^{\frac{1}{2}}}$$

where $\lambda_1 = \lambda/n_1$ is the wavelength in the denser medium and $n_{2-1} = n_2/n_1$ is the ratio of the refractive index of the rarer medium divided by that of the denser medium. The meaning of these relations is illustrated by Fig. 2 in which the penetration depth divided by the incident and reflected wavelength λ_1 is plotted versus the angle of incidence θ for a number of interfaces (i.e. for different ratios of n_2/n_1). It should be noted that the penetration depth is only about one-tenth the wavelength in the cases when the difference between the refraction indices is large, i.e. when n_2/n_1 is small, this being near the grazing angle ($\lambda = 90°$). This penetration becomes indefinitely large as λ approaches λ_c. At a fixed angle, the penetration depth is larger in the case of small index differences (i.e., as n_{2-1} approaches 1). The penetration depth is also proportional to wavelength and hence is greater at longer wavelengths. As an example if the dense medium is glass ($n_1 \cong 1.5$) and the rare medium is an aqueous analyte ($n_2 \cong 1.3$), $n_2/n_1 = 0.867$ which corresponds approximately to curve 11 of Fig. 2. In this case, the pene-

tration would be about 1/3 the wavelength at the grazing angle, theoretically infinite at the critical angle (60°) but already below 1 at 65°.

Since E decreases exponentially, the region beyond the boundary interface in which the amount of energy for interacting with the product is still significant corresponds to the depth z where the electric field magnitude is still a reasonable fraction of E_o, say a value of at least 0.1 E_o, better, the region in which E is between E_o and $E_o/3$. Thus, for optimum interaction efficiency, the thickness of the reaction product film should approximately match the depth of that region. This is illustrated on Fig. 3 which shows an incident (N) and a reflected beam (R), the zero depth vector E_o of the evanescent wave and a film of product reactant plus analyte (A), the thickness of which approximately matches the penetration depth d_p where E is about $E_o/3$. In Fig. 3, the influence of the refractive index of the thin film A is not considered significant because the thickness of this film does not exceed the depth of penetration of the evanescent wave. Indeed, the change of refractive index of the rare medium due to the growing of the analyte-reactant film in the reaction area is so small that the corresponding change of the value of the critical angle of reflection is practically negligible except for the reflecting modes quite close to that reflecting angle. Support to this view which constitutes an unexpected advantage of the invention over the prior art can be found for instance in the aforesaid N.Y. HARRICK reference, p.51.

Another point which should be emphasized for comparison with the prior art concerns the efficiency of the interaction of the light signal with the reaction product. In the classical spectrometric systems, a light signal is passed through a transparent holder containing the analyte (beaker or cuvette) and part of the energy is absorbed by the sample which leads to some degree of absorption that is measured. Yet, this method is not particularly efficient as the amount of analyte should be relatively large to provide significant interaction with the light signal under usual conditions. In contrast, in the present invention where the interaction of an evanescent wave with a film the thickness of which approximately matches with the penetration of that wave is involved, the efficiency is considerab-

ly higher since there is a strong light amplification effect in the interaction area. Indeed, as shown for instance in the previously mentioned HARRICK reference, the field strength of the evanescent wave within its range of interaction with the analyte-reactant layer is much stronger than that of the incoming signal. This is actually due to the simultaneous presence of both the incoming and outgoing beam field amplitudes.

The above discussed optical fundamentals useful for understanding the operating principles of the invention refer to the use of unpolarized light. In practice, it is important to note that the initial magnitude of the electric field at zero depth (E_o) is dependent on the state of polarization of the incident light wave. Thus, in some cases, polarized light in place of ordinary light can be advantageously used in practising the invention (it will be seen hereinafter that in case of measuring signal changes by ellipsometry, polarized light is essential) and, in such cases, the various optical parameters can be controlled and optimized for maximum response and sensitivity; for instance a selection of an appropriate incident polarization angle (e.g. polarization parallel or perpendicular to the plane of incidence) can be made for maximizing E_o.

In view of the above considerations, the following advantages of the invention relative to the prior art can be fully appreciated. Thus, in the case of a test involving one particular specific reactant for an analyte, the thickness of the product layer will usually be determined by the respective size of the product molecules. For instance, in a typical immunoassay, the product layer may be constituted of a first film of an antibody and a second film of an antigen. The thickness of this may range depending on the molecule types from several Angströms to several hundreds of Angströms or more. Now, in view of the thickness of the layer, the index of refraction of the core may be selected and also in some cases the wavelength so that the above discussed parameters will be matched as much as possible. To give an example by way of illustration, if the layer involved is relatively thin, cores with high refractive indices will be selected (for instance, sapphire, $n = 1.8$; silicon, $n = 3.4$) and, if compatible with the optical processes involved (i.e. absorption, scattering, fluorescence, etc..), shorter wavelengths will also be

selected. This will permit minimizing interaction of the evanescent wave with regions of the analyte solution deeper than the thickness of the space where the analytical reaction is taking place, thus minimizing the influence of undesirable extraneous factors (background noise, presence of impurities in the solution and the like). Obviously, none of the methods of the prior art can achieve such possibilities. It should also be kept in mind in appreciating the differences between the present invention and the prior art where the thickness of the sheath extends well beyond the penetration of the evanescent wave (whereby the refractive index (n_2) of the sheath becomes determinant in contrast with what happens in the invention) and in which n_2 is made greater than the index (n_1) of the core, that the light signal initially injected in the core and refracted into the sheath will not readily reenter into the core and be present at the output thereof as apparently believed by some (see for instance WO 81 100 912). Indeed, when a light signal is travelling in a rarer medium surrounded by a denser medium, refraction of said light signal into the sheath will occur. Then, this refracted wave will be totally reflected by the outside boundary of the sheath and will bounce back toward the core. Now, since the index n_1 of that core is smaller than that of the sheath, the wave will do either of the two following things: if the incident angle is larger than the critical angle, the wave will be again reflected and will stay definitely in the sheath. If the incident angle is smaller than the critical angle, the wave will go through the core and penetrate on the other side into the sheath and so on. So, in no case will a wave originally injected into the core and having been refracted in the sheath return solely in the core and be present therein at the output of the core unless it is still surrounded by the sheath. This is perfectly illustrated in Fig. 3B of USP 4,050,895. No shortcoming of that sort exists with the invention in which the key light signal only travels within the core and not in the outside layer containing the reactant and analyte.

The optical changes involved in the method of the invention can relate to different kinds of phenomena; for instance, the following phenomena can be involved: absorption of the light travelling in the core; scattering of the light signal by the reaction product; fluo-

rescence of the reaction product upon excitation by the light signal in the core. Further, the excitation signal in the core can be polarized and ellipticity polarization factors may be subject to modification by the analytical reaction and be monitored. Each of these possibilities are disclosed in more detail hereafter in this specification.

The types of analytical measurements which can be accomplished with the present method are so many that it is practically impossible to list them all. However, a few examples will be given hereinafter by way of illustration. However, before going any further in this direction, it is useful to develop somewhat the fundamentals pertaining to the application of the invention to "limited reagent" and "excess reagent" assays desirably used in biological and diagnostic analysis. For the purpose of such discussion, we shall conventionally call the analyte the "antigen" AG and the reagent the "antibody" AB. Needless to say that the reverse condition is also valid.

"Excess reagent" assay refers to cases in which an excess of a reactant in respect to the analyte is used. "Limited reagent" essentially involves the use of a system in which the test substance or analyte (containing the antigen to be measured) is treated with a limited amount of a specific reagent (the antibody) to give an analyte-reactant product (e.g. an AG.AB complex) plus some residual analyte. When the reaction is allowed to go to completion, i.e. if the assay proceeds to equilibrium ("saturation assay"), that is, the limiting conjugate reagent (AB) is saturated with the analyte, it is necessary to add, prior to the reaction (or, in sequential assays, at some time before the final equilibrium is reached, i.e. prior to measurement), a fixed amount of a labelled form of the analyte (AG^*) to the reaction mixture being under test. For the example of an antigen to be assayed with an antibody reagent, the proportion of the labelled antigen (AG^*) to the unlabelled one (unknown) shall stay the same in said residual analyte as it was at the start. Since the known amount of AB used will bind a known amount of the AG + AG^* mixture, it suffices to determine the residual AG^* or the AG^* bound to the AB (by means of its label) to calculate the amount of AG originally present in the sample. To give a simplified example, suppose that the sample contains x equivalents of an enzyme (AG) to be meas-

ured by means of a known amount g of an enzyme conjugate (AB) that forms an AG.AB complex (with, for instance, a 1:1 molecular ratio of both components). Then, prior to the reaction, a equivalents of the same enzyme to be measured but in labelled form (AG^*) are added to the sample. Thus, in the course of the reaction, a portion of g equivalents of antigen (AG + AG^*) is consumed by the g equivalents of antibody. Now, after removing the complex from the mixture, the residual AG^* is ascertained by conventional means. If it is found, by substracting the value measured for the remaining AG^*, that the amount actually used up was b equivalents, it becomes evident, since AG and AG^* are chemically indentical and consumed at the same rate, that the ratio of consumed AG^* to consumed AG, i.e. b/g-b should be equal to the original ratio a/x, from which $x = a(g-b)/b$ can be calculated.

This type of approach is quite attractive although, in the prior art applications, it suffers from some drawbacks, one of them being the general requirement that the complex (mixture of AG^*.AB + AG.AB) must be separated from the reaction medium which is sometimes tedious and a possible source of errors. Now, when applied to the present invention, this drawback is non-existent because the complex that forms automatically removes the analyte from the solution as it deposits onto the wave-guide. To illustrate the application of the present method to "saturation type assays" one shall again begin with a wave-guide, say an optical fiber coated with a specific antibody AB which is immersed into a buffer solution and allowed to equilibrate with it. The unknown amount of complementary antigen AG to be determined is then added as before but simultaneously with a known small amount of the same antigen labelled with a molecule having specific optical properties (AG^*) e.g. optical absorption, fluorescence, etc..) which may be detected by the evanescent wave interaction at the surface of the coated fiber using suitable optical arrangements to be described in this specification. Now, since both labelled and unlabelled AG are essentially identical in reactivity towards the AB-coated fibre optic, but only the labelled species can be detected via its label, the apparent change in the optical property detected through the fibre (e.g. fall in absorbance if an absorbing label is used) will be inversely proportional to the unknown concen-

tration of AG and can be determined with reference to a suitable series of known standards. This kind of application is illustrated in one of the Examples hereinafter.

At this point it is useful to make a clear statement concerning the label used to measure the reaction that takes place. Normally, in immunoassays, it is not possible to measure the analyte directly because the concentrations of analytes and reagents are extremely low. Since the equilibrium mixture in limited reagent assays essentially only contains excess analyte and a fixed amount of bound complex, when the former cannot be measured directly and the latter is a fixed amount, no quantitative estimation of the original analyte concentration can be obtained even after separation of the complex and the excess antigen. The added labelled tracer (a small quantity of labelled analyte) is necessary to allow measurement via the label according to its distribution between the bound moiety and the free moiety.

If the analyte, however, has an intrinsic property that can be detected when it is concentrated locally (i.e. in situ separation and in situ concentration), e.g. on the surface of a fiber optic probe, then the addition of a labelled analyte tracer is no longer necessary. Thus, in the present invention, the in situ concentration of the analyte-reagent complex may allow for the detection of the analyte without resort to a labelled tracer. However, local concentration of the analyte reagent complex (since the amount of reagent is fixed) will not allow the quantitative determination of the original analyte concentration unless the reaction between analyte and reagent is measured kinetically or unless the total amount of analyte is less than the number of reagent bonding sites. Thus, using the in situ separation and concentration of the complex bound analyte in conjunction with a sensitive detection system in the kinetic mode as in the present invention allows quantitative detection of the analyte in limited reagent system without resort to a labelled tracer.

As far as labelled systems are concerned, a distinction can be drawn between tracer systems in which a labelled version of the analyte is added in trace amounts to the reaction and the labelled reagent systems in which a label is attached to the specific reagent.

The former tracer reagent is normally used in limited reagent assays (e.g. standard radio immunoassays), while the latter is normally used in excess reagent assays (e.g. standard sandwich assays) and can be used in certain forms of kinetic reagent assays (e.g. KRONICK et al, USP 3,939,350); labelled analyte and labelled reagent systems are suitable for the present invention if the label used is capable of being detected in an optical wave guide system (e.g. absorbing or fluorescent labels).

For the next part of the discussion, reference will be made to another part of the annexed drawing in which

Fig. 4 is a diagram illustrating a first type of assay called "direct type of assay".

Fig. 5 is a diagram illustrating a competitive "limited reagent" type of assay.

Fig. 6 is a diagram illustrating an indirect competitive "limited reagent" type assay.

Fig. 7 is a diagram illustrating a sequential "saturation" type assay.

Fig. 8 is a diagram illustrating a "sandwich" type assay.

The most straightforward case of assay to which the invention is applicable is schematized on Fig. 4. This is called the "direct" type assay. In this assay, a wave-guide core 1 of which only a portion (with refractive index n_1) is represented is provided with a film of antibody AB and this core is immersed in an analyte solution having refractive index n_2 ($n_1 > n_2$) containing an antigen AG? to be determined. The antigen will attach to the AB molecules at a rate proportional to the antigen concentration [AG?] (since the amount of AB film on the core is a fixed entity) and when this rate is determined, it can be correlated with standard rates obtained from calibrating AG solutions and [AG?] can be determined. So, the amount of AB available can be "limited" or it can be in excess and the reaction can go to an equilibrium. For detecting the AB-AG complex formation by means of the optical changes occurring in the core, the various aforesaid techniques can be used (i.e. extinction of the signal by absorption, scattering and fluorescence phenomena, etc..), provided the formation of AB.AG generates the required optical changes. So, the test applies particularly well to large molecules able